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Protein Profiles of Pod Borer Maruca Resistant Transgenic Cowpea

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Abstract

The grain legume cowpea *Vigna unguiculata* (L.) Walp. is a major protein source used for food and feed in Sub-Saharan Africa. The crop is affected by the pod borer *Maruca vitrata* against which transgenic lines were developed as part of the genetic control approach. This study aimed to assess the protein profiles in seeds and leaves of transgenic cowpea lines and their non-transgenic near-isogenic counterparts. Crude protein content was determined by the Kjeldahl method, and soluble proteins were quantified using Bradford dye binding assay. The average crude protein content ranged between 21.61% and 26.58% in the seeds and between 10.86% and 17.90% in the leaves. Total solubility varied between 13.03% and 20.64%. Osborne's protein fractions contents in the seeds were 52.41% - 69.52% (albumin), 4.62% - 7.19% (globulin), 7.95% - 11.40% (glutelin) and 3% - 4% (prolamin). In any case, protein content differed significantly between cowpea genotypes but not between pairs of transgenic/non-transgenic lines. Insecticidal Cry1Ab protein expressed by transgenic lines was only detected in the albumin and globulin fractions. Altogether, these findings enhance our understanding of the effects of genetic modification on cowpea protein content and composition, with potential implications for nutritional and safety assessments.

Keywords

Cowpea, Protein, Cry1Ab, Protein Fractions

1. Introduction

Cowpea [*Vigna unguiculata* L. (Walp.)] is the most important grain in the dry savannahs of Africa where it originated [1] [2]. It is widely grown and in many tropical and subtropical areas in Southeast Asia, the southern United States of America and Latin America. According to the Food and Agriculture Organization (FAO) statistics for 2021 [3], Africa contributed to nearly 97% of the world's cowpea production. The main producers in Africa were Nigeria (3.63 million tons), Niger (2.66 million tons) and Burkina Faso (0.71 million tons). Cowpea production in these three countries accounted for 80% of cowpea produced in Africa.

Cowpea is used for both food and feed due to its relatively high content in essential amino acids-rich proteins [4]. As for other leguminous crops, cowpea seed proteins consist of four fractions (albumin, globulin, prolamin and glutelin) with different solubility characteristics [5] [6]. Due to its richness in proteins, cowpea is a good supplement for a low-protein diet based on cereals and tuber crops [7]. Besides its nutritional use, cowpea is a leguminous plant with nitrogen fixation ability, therefore contributing to soil quality [8].

Cowpea is known to be a resilient crop that can tolerate low rainfall and poor soil conditions. However, its production is heavily affected by several pests and diseases. The legume pod borer *Maruca vitrata* Fabricius is considered a pantropical legume pest and the most damaging cowpea pest in West Africa [9]. Many commonly used approaches for controlling insect pests, including the use of pesticides, cultural management and host plant resistance had limited success with *M. vitrata* [10]. While new pesticides and the use of parasitoids are envisaged for more effective control, attempts to develop genetically engineered pod borer-resistant (PBR) cowpeas harboring the Cry1Ab gene were successful [10]. Selected PBR cowpea lines were used for Cry1Ab gene introgression into a few non-transgenic cowpea cultivars through conventional breeding. The non-transgenic cultivars were chosen on the ground that they were already improved for some traits such as striga resistance and grain quality and released to farmers. Cry1Ab toxin expressed from the Cry1Ab gene was the only new trait introduced in non-transgenic cultivars. It was just meant to protect the cowpea plants from insect damage under *M. vitrata* infestation [10].

Compositional assessment of food and feed comparing the composition of genetically engineered crops to their respective parental cultivars in relation to important nutrients and toxicants is a requirement in genetically modified organisms regulation [11]. Such information is part of the elements used to assess the intended and particularly, the potential unintended changes in the plant be-

cause of the genetic modification. This study focused on proteins as key nutrients in cowpeas. The study aimed to compare protein contents and proteins fractions in seeds and dead leaves of some PBR cowpeas and their respective conventional parents.

2. Materials and Methods

2.1. Plant Materials

Four transgenic cowpea lines referred to as IT97K-T, IT98K-T, Gourgou-T and Nafi-T were used along with their respective non-transgenic lines (IT97K, IT98K, Gourgou and Nafi) were used. The respective transgenic and non-transgenic lines (also known as conventional lines) had similar genetic make-ups, except the presence of the cry1Ab gene in transgenic lines. All seeds were taken from the National Biosafety Laboratory seed collection and were originally obtained from the Institut de l'Environnement et de Recherches Agricoles, Burkina Faso, as part of the biosafety regulatory process.

Seeds from transgenic and conventional cowpeas were sown in 10-liter buckets in the greenhouse. One week after germination, a leaf sample of about 0.1 gm was collected from each plant for Cry1Ab protein detection to ascertain the transgenic or conventional status of the cowpea plants.

2.2. Serological Detection of Cry1Ab Protein Seed Lots and Plant Leaves

The presence or absence of Cry1Ab in seeds of transgenic or conventional cowpea were tested for quality control. The seeds were tested individually by enzyme-linked immunosorbent assay (ELISA) using the QualiPlate kit for Cry1Ab/Cry1Ac (Envirologix Inc., USA) according to the manufacturer's instructions. Each seed was crushed on a piece of paper using a pestle. A portion (~0.1 gm) was collected and finely ground in 1 mL extraction buffer (phosphate buffer saline, pH 7.4 containing 2% of polyvinylpyrrolidone). Seed extracts were then centrifuged at 10,000 x g for 10 min and the supernatants were used as antigen sources. Known non-transgenic seeds were used as negative controls. The threshold for Cry1Ab detection was determined as the average absorbance readings from negative controls plus three times the standard deviation [12]. For each cowpea line, the rest of Cry1Ab-positive seeds were pooled, ground in a coffee grinder, and passed through a 75 µm sieve.

Antigen sources for the leaves were obtained by grinding 0.1 gm in 1 mL of extraction buffer using an MP FastPrep 24 homogenizer. Leaf extracts were subsequently treated as indicated for the seed extracts.

2.3. Extraction of Seed Proteins

Soluble proteins were extracted in sodium sulfate buffer (50 mM, pH 7.8) as described by Hameed *et al.* [13] and Saminu and Muhammad [14]. The flour (1 gm) was homogenized in 10 ml of buffer and centrifuged at 10,000 x g and 4˚C. The supernatant was used for protein quantification. Total crude protein content

was determined from seed flour using the Kjeldahl method with an automatic Kjeldahl analyzer UDK159 (VELP Scientific, Italy) and a conversion factor of 6.25 [15].

The Osborne protein fractions were extracted sequentially in different solvents as described earlier [5]. Briefly, the albumin fraction was extracted by mixing 3.5 g of seed flour and 50 mL of distilled. The mixture was vortexed briefly, agitated on a rotary shaker for 30 min at room temperature and centrifuged at 10,000 x g for 10 min. The supernatant was collected, and pellet was extracted once more. The supernatant of the two extraction cycles was pooled to form the albumin fraction. The pellet from the last centrifugation was used to extract the next protein fraction. Thus, globulin, prolamin and glutelin fractions were successively extracted in a similar manner using 1M NaCl, 70% (v/v) ethanol and 0.2% NaOH, respectively. Quantitation of protein fractions was done in the supernatants using Bradford's dye binding assay [16] with the following modification to solve the non-linearity issue of the calibration curve [17]: absorbances of the protein-dye complex were recorded at 450 nm and 590 nm. Bovine serum albumin was used as a reference protein. Protein fractions were tested by ELISA to identify the ones containing the Cry1Ab protein, using the QualiPlate kit for Cry1Ab/Cry1Ac (Envirologix Inc., USA) according to the manufacturer's instructions.

2.4. Quantitation of Proteins in Leaves

Soon after harvesting, the leaves were collected from both transgenic and conventional plants for protein quantitation in the leaves. Leaf samples were dried for one week at 50˚C and were used for the determination of crude protein content using the Kjeldahl method [15].

2.5. Statistical Analysis

Absorbance data were collected using Microsoft Excel and subsequently transferred in R software [18] for all statistical analyses. Average protein contents in cowpea lines were presented as mean ± standard deviation of three replicates. Data were checked first for distribution normality using QQ-plots and the Shapiro-Wilk test and for homogeneity of variances using the Levene test. Then, analysis of variance (ANOVA) was used to compare mean protein contents in cowpea lines. Post-hoc tests for separation of the means were done using Fisher's LSD at $p < 0.05$.

3. Results

3.1. Crude Protein Content in Cowpea Seeds

Figure 1 shows the crude protein content of the seeds of transgenic and non-transgenic cowpea lines. The protein content (%) varied between 21.61 ± 0.09 and 26.58 ± 0.08. Statistical analyses showed significant differences between protein content in the cowpea lines ($F_{7,16} = 337.1$; $p < 0.001$). The cowpea line

IT98K-T showed the highest protein content (26.58% ± 0.08%) which differed significantly from the protein content in its non-transgenic counterpart IT98K ($p < 0.001$). No significant differences were found between the other pairs of transgenic/non-transgenic lines: IT97-T/IT97 ($p = 0.753$), Gourgou-T/Gourgou ($p = 0.108$) and Nafi-T/Nafi ($p = 0.681$). All these three pairs differed significantly from one another. Altogether, the average protein content in transgenic and non-transgenic cowpea lines were 23.84% ± 2.03% and 22.79% ± 1.09%, respectively. The Welch two-sample t-test showed no significant difference ($t = 1.57$, $p = 0.135$).

3.2. Total Soluble Protein Contents in Cowpea Lines

Total soluble protein extracted in sodium phosphate buffer ranged between 13.03% and 20.64%. Significant differences were found between cowpea lines as indicated by the Kruskal-Wallis test [H (7) = 28.71, p = 0. 0002]. A large effect size was detected, eta2[H] = 0.34. This indicated that 34% of the variance in the protein content was explained by the variable "cowpea line". Pairwise Dunn's test showed significant differences between a few pairs of cowpea lines *i.e.* IT97K-T/Nafi-T, IT97K/Nafi-T, Gourgou-T/Nafi_T and Gourgou/Nafi-T (Figure 2). No significant difference was found between pairs of any transgenic cowpea line and its conventional counterpart.

3.3. Crude Protein Content in Cowpea Leaves

The crude protein contents in cowpea leaves are illustrated in Figure 3. The highest and lowest protein content were recorded in Gourgou-T (17.90% ± 0.56%) and Nafi-T (10.86% ± 0.61%), respectively. Significant differences were found between cowpea lines ($F_{7,16} = 162.5$; $p < 0.001$). All transgenic/non-transgenic pairs of cowpea lines differed from one another.

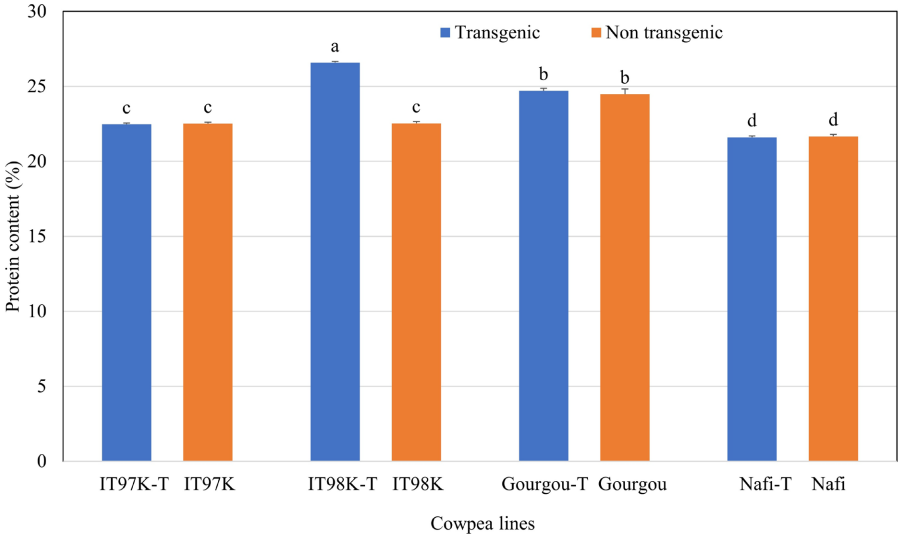

Figure 1. Crude protein content in transgenic and conventional cowpea seeds. Identical letters on the top of the bars indicate groups with non-significant differences in crude protein content.

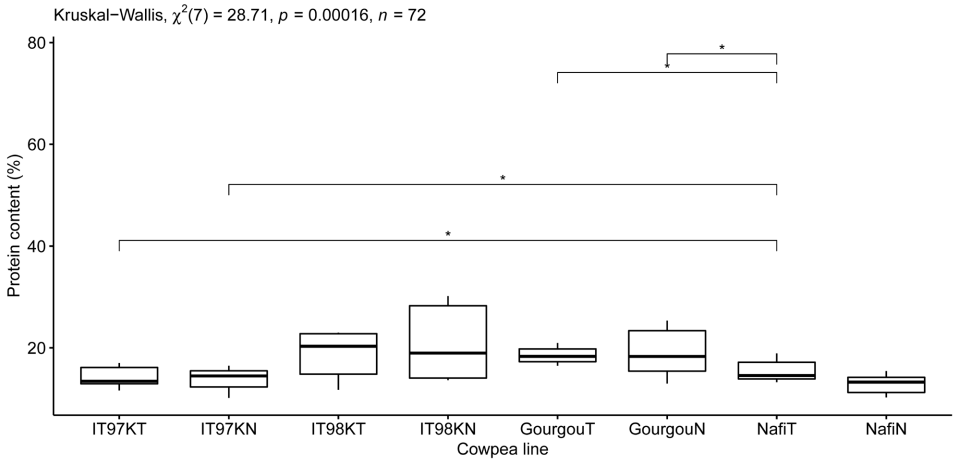

Figure 2. Total soluble protein content in cowpea seeds. Each data point represents three independent extractions, each tested in triplicates. Identical letters on the top of the bars indicate groups with non-significant differences in crude protein content.

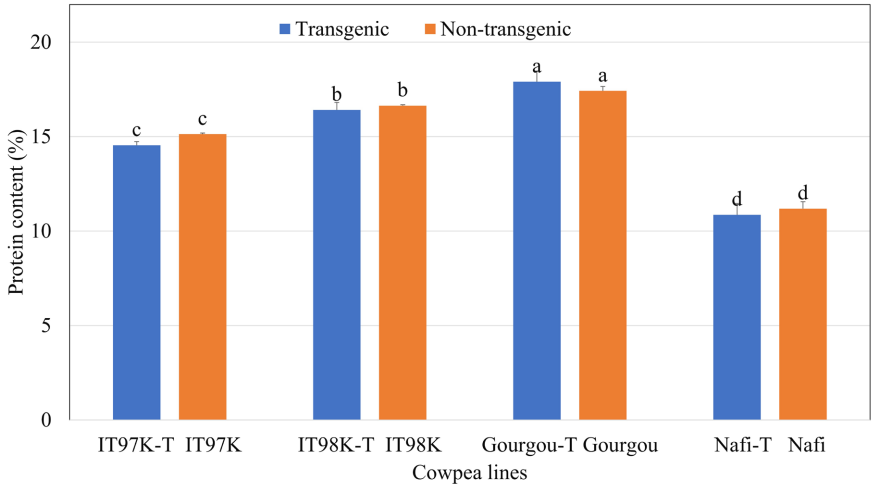

Figure 3. Crude protein content in cowpea leaves. Identical letters on the top of the bars indicate groups with non-significant differences in crude protein content.

However, no significant difference was found between cowpea lines in the same pair, indicating similar protein contents between transgenic cowpea lines and their non-transgenic counterparts. Taken together, protein contents in transgenic (14.93% ± 2.78%) and non-transgenic (15.10% ± 2.52%) cowpea lines did not differ significantly, as indicated by the Wilcoxon test ($p = 0.80$).

3.4. Protein Fractions Contents in Cowpea Lines

The results of the determination of the protein fractions contents in cowpea lines are summarized in **Table 1**. The albumin fractions content varied between 52.41% ± 2.86% and 69.52% ± 4.50% of total protein. As revealed by one-way ANOVA, albumin content was significantly different between cowpea lines ($F_{7,16} = 12.5$, $p < 0.001$). However, pairwise comparisons indicated that no significant differences were observed between any pair of transgenic/non-transgenic cowpea

Table 1. Osborne protein fractions contents in cowpea lines.

Cowpea line[a]	Total protein	Osborne protein fractions (% total protein)				% recovery
		Albumin	Globulin	Prolamin	Glutelin	
IT97K-T	22.47 ± 0.09 c	54.12 ± 1.87 ef	7.07 ± 0.23 a	3.47 ± 0.22	8.56 ± 0.17 bc	73.34 ± 2.35 ef
IT97K	22.51 ± 0.11 c	52.41 ± 2.86 f	6.25 ± 0.55 ab	3.58 ± 0.33	7.95 ± 0.66 c	69.66 ± 3.53 f
IT98K-T	26.58 ± 0.08 a	60.74 ± 3.33 cd	6.71 ± 0.65 a	3.05 ± 0.23	8.51 ± 1.06 c	79.23 ± 4.32 bcd
IT98K	22.53 ± 0.12 c	64.26 ± 2.73 bc	7.19 ± 0.44 a	3.59 ± 0.53	8.36 ± 0.53 c	83.58 ± 2.28 abc
Gourgou-T	24.70 ± 0.17 b	59.82 ± 2.42 cd	5.38 ± 0.65 bc	3.77 ± 0.28	8.74 ± 0.56 bc	77.53 ± 3.09 de
Gourgou	24.47 ± 0.35 b	58.61 ± 1.86 de	5.44 ± 1.05 bc	3.26 ± 0.28	10.8 ± 1.39 a	78.31 ± 1.04 cde
Nafi-T	21.61 ± 0.09 d	66.14 ± 2.30 ab	4.39 ± 0.80 c	3.56 ± 0.47	10.3 ± 1.57 ab	84.03 ± 3.54 ab
Nafi	21.66 ± 0.14 d	69.52 ± 4.50 a	4.62 ± 0.52 c	3.18 ± 0.29	11.4 ± 1.35 a	89.15 ± 4.66 a

[a]Transgenic cowpea lines were derived from those of their respective conventional near-isogenic lines by adding "-T". Means followed by the same letter(s) within each column are not significantly different. Cowpea lines did not differ significantly in prolamin content.

line. Globulin and glutelin contents were considerably lower than albumin and varied in the respective ranges of 4.62% ± 0.52% - 7.19% ± 0.44% and 7.95% ± 0.66% - 11.40% ± 1.35%. As for the albumin fraction, cowpea lines differed significantly in globulin ($F_{7,16} = 8.25$, $p < 0.001$) and glutelin ($F_{7,16} = 4.92$, $p = 0.004$) contents. No significant difference was observed between cowpea lines of the same transgenic/non-transgenic pair. Prolamin content in all was low in all cowpea lines, varying in a narrow range of around 3% - 4%. Consequently, no significant differences were found between the cowpea lines ($F = 1.50$; $p = 0.24$).

3.5. Cry1Ab Detection in Protein Fractions

The Cry1Ab protein was clearly detected in albumin and globulin fractions (**Figure 4**). Absorbance readings were far above the detection threshold. Mean absorbance readings in the albumin fraction were 2.85 times that in the globulin fraction, indicating a much higher presence of Cry1Ab protein in the former fraction. No detection of the protein was achieved in the prolamin and glutelin fractions. As expected, Cry1Ab protein was not detected in protein fractions from non-transgenic cowpea cultivars.

4. Discussion

Proteins are key metabolites that are often targeted in assessing unintended effects of gene modifications [19]. In this study, the protein profile of four Cry1Ab-expressing transgenic cowpea lines was compared to that of their respective near-isogenic conventional counterparts. Proteins are major cowpea nutrients giving the crop all its importance as food and feed. Crude protein content in the seeds of cowpea lines varied between 21.6% and 26.6%. These results are consistent with those of most studies which reported protein contents in the range of 20% to 30% [5] [6] [20] [21]. Lower values down to 15.06% and

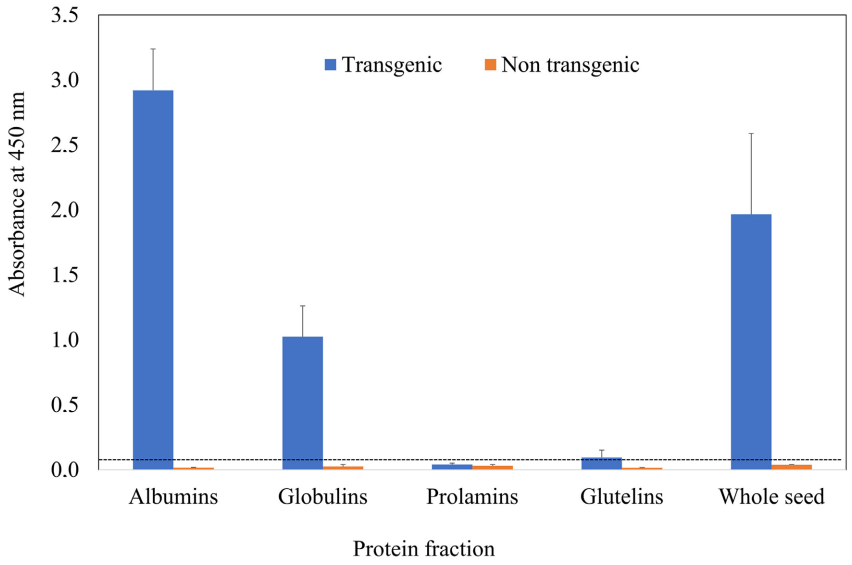

Figure 4. Detection of Cry1Ab protein in protein fractions of transgenic and conventional cowpea lines. Whole seeds were used as controls. Data are absorbance readings averaged over all cowpea lines and error bars represent the standard deviations. The dashed line indicates the detection threshold.

higher values up to 40% were also reported [22] [23]. Variation in protein content was mainly attributed to cowpea genotype [23]. The protein content within all but one pair of transgenic and non-transgenic cowpea lines did not differ significantly. Similar results were found for Cry1Ab expressing maize [24] [25] [26]. Therefore, the significant difference in protein content between IT98K-T and IT98K lines is possibly due a specific intra-plant interactions with the Cry1Ab gene or to other factors than the sole presence of the gene. The variation in protein content between cowpea lines was also reflected in the soluble protein portion. However, differences in protein content between pairs of corresponding transgenic and non-transgenic cowpea lines were not significant.

The crude protein content in cowpea leaves (10.86% - 17.90%) was found significantly lower than the range of 23% - 40% reported earlier [23]. The lower protein content is likely due to the after-harvest stage of leaf sample collection when cowpea leaves were senescent or drying. Usually, cowpea leaves and other plant debris are collected after harvest to be used as feed. Interestingly, no significant differences were observed between transgenic and non-transgenic cowpea lines belonging to the same pairs.

A more detailed assessment of protein content in the seeds indicated that albumin fraction largely dominated over all other fractions. Similar results were found by other authors in cowpea [5], amaranth [27] and cumin [28]. Conversely, other studies indicated that globulins were the major protein fraction in cowpeas [6] [29] [30]. Discrepancies between these studies were likely due to the order of extraction of protein fractions. The albumin fraction tended to be dominant when extraction was first done in water. By contrast, the salt-soluble globulin fraction dominated when the first extraction was done the salt solution.

Noticeably, no significant difference was found between transgenic cowpea and their corresponding near-isogenic non-transgenic ones, whatever the protein fraction. This suggested that the protein fraction contents were not affected by the genetic modification.

The Cry1Ab protein expressed by transgenic cowpea lines was found in the albumin and globulin fractions. The notably higher presence of Cry1Ab protein in the albumin fraction suggested a preferential association of this transgenic protein with albumins and highlighted its rather water-soluble properties.

5. Conclusion

The protein profiles of Cry1Ab-expressing cowpea lines were assessed along with those of corresponding near-isogenic non-transgenic lines. This study revealed that genetic modification had a limited impact on the overall protein content in seeds and leaves. While some significant differences were observed in protein content and composition among cowpea lines, these variations were primarily between distinct cowpea genotypes. Altogether, corresponding transgenic and non-transgenic pairs of cowpea lines displayed similar protein profiles, aligning with the expected outcomes of the genetic modification. These results enhance our understanding of the effects of genetic modification on cowpea protein content and composition, with potential implications for nutritional and safety assessments.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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